Antiinflammatory Mediator Lipoxin A4 and Its Receptor in Synovitis of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To evaluate the role of an antiinflammatory lipid mediator, lipoxin A_4 (LXA_4), in inflammatory arthritis, we measured the level of LXA_4 in synovial fluid and lipoxin A_4 receptor (ALX) expression in synovial tissues obtained from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods. Levels of LXA_4 and its analog (15-epi-LXA_4) in synovial fluid from 30 patients with RA and 15 patients with OA were measured by a specific ELISA. Reverse transcription-polymerase chain reaction (RT-PCR), real-time quantitative PCR, and *in situ* hybridization were performed to detect mRNA for ALX and 15-LOX, and LXA_4 synthetase, in synovial tissues from 20 patients with RA and 10 patients with OA.

Results. Both LXA_4 and 15-epi-LXA_4 showed significantly higher levels in RA synovial fluid (10.34 ± 14.12 ng/ml for LXA_4) than OA synovial fluid (0.66 ± 0.77 ng/ml for LXA_4). Logarithmic concentration of LXA_4 was significantly correlated with that of leukotriene B_4 and prostaglandin E_2 in RA and OA synovial fluids. Expressions of ALX and 15-LOX mRNA were stronger in RA synovium than OA synovium. Expression of mRNA for interleukin 13 (IL-13), which induces 15-LOX, was significantly stronger in RA synovium than OA synovium.

Conclusion. ALX is an important target of LXA_4 in synovial tissues of patients with RA. 15-LOX induced by IL-13 might regulate the production of LXA_4 to have an antiinflammatory effect against proinflammatory lipid mediators in inflamed joints. These findings could lead to the development of new therapy for inflammatory arthritis such as RA. (First Release Oct 1 2007; J Rheumatol 2007;34:2144–53)

Key Indexing Terms:
RHEUMATOID ARTHRITIS   LIPOXIN A_4 LIPOXIN   INFLAMMATION

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Lipoxin A_4 (LXA_4) and its recently identified carbon-15 epimeric form, aspirin-triggered LXA_4 (15-epi-LXA_4), potently inhibit neutrophil activity and appear to serve as an endogenous "stop signal" that regulates excessive leukocyte trafficking and promotes resolution of inflammation[1]. LXA_4 is synthesized by 5-lipoxygenase (LOX) and 12-LOX, or by 5-LOX and 15-LOX, via cell–cell interactions, while 15-epi-LXA_4 is produced by 5-LOX and acetylated prostaglandin H synthase-II after treatment with aspirin. Both 15-epi-LXA_4 and LXA_4 modulate leukocyte responses by interacting with the lipoxin A_4 receptor (ALX), which is a specific G-protein-coupled receptor[2]. ALX was first identified in retinoic acid-differentiated HL-60 cells and then was cloned in mice, showing a high affinity to its endogenous lipid ligands (LXA_4 and 15-epi-LXA_4) as well as their stable bioactive analogs[3]. These compounds inhibit acute inflammation and reperfusion injury in both human cell models and murine models[4-6].

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by inflammatory polyarthritis and is associated with higher levels of proinflammatory arachidonic acid metabolites, such as leukotriene B_4 (LTB_4)[7] and prostaglandin E_2 (PGE_2)[8], in the synovial fluid than in that of patients with

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osteoarthritis (OA). 5-LOX is expressed in synoviocytes[9] and the synovial lining layer[10] of patients with RA. Neutrophils in the synovial fluid[11] and peripheral blood[12] of patients with RA generate more LTB_4 via upregulated 5-LOX than the same cells from healthy individuals. PGE_2 induces 15-LOX in human neutrophils[4], while 15-LOX was reported to be upregulated by interleukin 4 (IL-4)[13] and IL-13[14] in human monocytes. 15-LOX is a key enzyme of LXA_4 synthesis in inflammatory sites[4]. An earlier study showed that polymorphonuclear cells from patients with RA can release more lipoxins than those from healthy individuals[15] and another exhibited the production of LXA_4 and 15-epi-LXA_4 by OA cartilage explants and ALX on OA chondrocytes[16,17]. These studies suggest the relationship between the lipoxin system and the pathogenesis of RA and OA.

We investigated LXA_4 in synovial fluid, as well as the differences of ALX distribution and expression in synovial tissues between patients with RA and OA. Factors that may regulate the lipoxygenase pathway were also analyzed to assess the role of LXA_4 and its analogs in the pathogenesis of RA.

MATERIALS AND METHODS

Reagents. A mouse anti-5-LOX monoclonal antibody was purchased from Research Diagnostic Inc. (Flanders, NJ, USA). The anti-CD3 antibody, anti-CD4 antibody, anti-CD8 antibody, and anti-CD68 antibody, and anti-von Willebrand factor (vWF) antibody were all purchased from Dako (Carpinteria, CA, USA). A DIG-High Prime DNA Labeling and Detection Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany) and a Vectorstain ABC kit came from Vector Laboratories Inc. (Burlingame, CA, USA). Finally, 3,3'-diaminobenzidine (DAB) was obtained from Dojindo (Kumamoto, Japan) and a real-time quantitative polymerase chain reaction (PCR) kit (qPCR Mastermix for Sybr Green 1) was purchased from Eurogentec (Seraing, Belgium).

Patients and samples. Synovial fluid specimens were obtained by arthrocentesis of the knee joint in 30 patients with RA and 15 with OA who consulted our institution (Table 1). Synovial tissue samples were obtained from 20 patients with RA and 10 with OA during orthopedic procedures for treatment at our institution. All patients with RA fulfilled the criteria of the American College of Rheumatolgy[18,19]. OA was diagnosed according to clinical and radiological criteria. All patients gave informed consent for the use of their samples in research. In both groups, the clinical characteristics of the patients (sex, age, C-reactive protein, and therapy) were consistent with the diagnosis.

Table 1. Clinical features of 45 patients with RA or OA used in the analysis of synovial fluid.

Feature	Diagnostic Group RA	Diagnostic Group OA
Patients, n	30	15
Female:male	22:8	10:5
Age, yrs	57.23 ± 13.30	66.07 ± 12.20
CRP, mg/dl	3.037 ± 1.889	0.2256 ± 0.2308
Medication		
PSL, n (%)	27 (90)	0 (0)
PSL, mean ± SD (mg/day)	4.724 ± 2.658	0 ± 0
NSAID, n (%)	25 (83)	6 (40)
Aspirin, n (%)	4 (13)	1 (7)
Statin, n (%)	0 (0)	1 (7)

CRP: C-reactive protein; PSL: prednisolone; Statin: HMG-CoA reductase inhibitor; NSAID: nonsteroidal antiinflammatory drugs.

Samples were fixed in 4% paraformaldehyde within 6 h of resection and were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) after cooling in liquid nitrogen. Synovial fluid cells were obtained from the synovial fluid samples of patients with RA, and the percentages of neutrophils and mononuclear cells were evaluated by examination after Wright-Giemsa staining.

Measurement of LXA_4, LTB_4, and PGE_2 in synovial fluid. Synovial fluid samples were collected in polypropylene tubes and centrifuged at 1800 g for 10 min at 4°C. Cell-free supernatants of the fluid were stored at –70°C until use. LXA_4, LTB_4, and PGE_2 were separated from the supernatant by passage through octadecylsilyl silica columns (Sep-Pak C18, Waters Corp., Milford, MA, USA), followed by elution with methyl formate for LTB_4 and LXA_4, or with ethyl acetate containing 1% methanol for PGE_2. After evaporation to dryness, the residue was resuspended in the extraction buffer of each ELISA kit. The LXA_4 level in synovial fluid was determined with an ELISA kit (Neogen Corp., Lexington, KY, USA) according to the manufacturer's instructions, which was specific for LXA_4 and showed little cross-reactivity [LXA_4 100%, lipoxin B_4 1.0%, 15-hydroxyeicosatetraenoic acid (HETE) 0.1%, 5-HETE < 0.1%, and 12-HETE < 0.1%]. LTB_4 and PGE_2 were also measured in the same samples of synovial fluid with specific ELISA kits (LTB_4, Neogen Corp.; PGE_2, Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturers' instructions.

Assessment of human LXA_4 receptor (ALX), 15-LOX, and IL-13 mRNA expression in synovial tissue by reverse transcription-PCR (RT-PCR) and real-time quantitative PCR. Total RNA was isolated from tissues or synovial fluid cells using the guanidium thiocyanate/phenol/chloroform method (Isogen Reagent Kit; Nippon Gene Co. Ltd., Toyama, Japan), and cDNA was synthesized from 2 µg of total RNA using RAV2 reverse transcriptase and Oligo (dT) primers (Takara Shuzo Co. Ltd., Shiga, Japan), as described[20].

The RT-PCR primers for human ALX (GenBank accession no. U81501) were 5'-CTG GCC CTG GCT GAC TTT TCT TT-3' (sense: 219–241 bp) and 5'-GCC ACC TTC AGC CTC TCC TCA-3' (antisense: 581–601 bp), and the PCR product obtained with these primers was 383 bp in size[21]. The primers for human 5-LOX (GeneBank accession no. J03571) were 5'-CCG GCA CTG ACG ACT ACA TCT A-3' (sense: 81–102 bp) and 5'-CAC GGG GGT AAA TCC TTG TGG-3' (antisense: 514–533 bp), and the PCR product had a size of 453 bp[9]. The primers for human 15-LOX (GenBank accession no. M23892) were 5'-TGG CCG ACC TCG CTA TCA AAG ACT-3' (sense: 548–571 bp) and 5'-TGG GGG ATC CGT AGG CAA GAA AAG-3' (antisense: 991–1014 bp), and the PCR product had a size of 467 bp[22]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. M33197) was used as the internal control, with 2 primers (5'-CAT CAT CTC TGC CCC CTC TG-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3') yielding an expected PCR product of 437 bp[23].

Real-time quantitative PCR was performed to compare the expression of ALX, 15-LOX, IL-4, and IL-13 mRNA in the synovial tissue samples obtained from patients with RA and OA. cDNA from 20 patients with RA and 10 with OA was subjected to real-time quantitative PCR with the following primers: 5'-AAC CCC ATG CTT TAC GTC TTT GTG-3' (sense: 912–935 bp) and 5'-ATT GGC AGC CGT GTC ATT AGT TG-3' (antisense: 1012–1034 bp) for human ALX[21], yielding a product of 123 bp; and 5'-ACC AGC CCC AGC AAG AGC ACA AG-3' (sense: 1081–1103 bp) and 5'-TTC AAG GGG TCT ACA TGG CAA CTG-3' (antisense: 1180–1203 bp) primers for human GAPDH (the control) yielding a product of 123 bp[23]. The primers for human 15-LOX were 5'-CCG GAT TTT CTG GTG TGG TTC-3' (sense: 615–634 bp) and 5'-ACT AGG CGA GCA GGA AGG TGA-3' (antisense: 738–758 bp), and the PCR product had a size of 144 bp[22]. The primers for human IL-4 (GenBank accession no. BC066278) were 5'-TCT GTG CAC CGA GTT GAC C-3' (sense: 203–221 bp) and 5'-ACC CAG GCA GCG AGT GT-3' (antisense: 322–338 bp), and the product had a size of 136 bp[24]. The primers for human IL-13 (GenBank accession no. NM_002188) were 5'-GGC CCT GAG CTC GGT GGA C-3' (sense: 679–697 bp) and 5'-CTA CAC

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CCC TCC CCT GCC CTA-3' (antisense: 715–735 bp), with the product having a size of 57 bp[25].

Real-time PCR was done with a real-time quantitative PCR kit (qPCR Mastermix for Sybr Green I, Eurogentec) according to the manufacturer's protocol. Detection was performed by identifying the fluorescence of SYBR Green fluorescent dye (Eurogentec). Amplification was performed according to the standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles of 95°C for 15 s each, and annealing for 1 min at 56.9°C for ALX, 56.7°C for 15-LOX, 54.6°C for IL-4, and 55.5°C for IL-13). All samples were measured in duplicate. Analysis was carried out with an ABI Prism 7700 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan), and the calculated cycle threshold values (Ct) were exported to Microsoft Excel. For comparison between the sample groups, relative mRNA levels were subsequently normalized against values found in the patients with OA, which were defined as the baseline (reference value = 1).

In situ hybridization of ALX and 15-LOX in synovial tissue. PCR products of ALX and 15-LOX were purified from agarose gels using a StrataPrep PCR purification kit (Stratagene Cloning Systems, La Jolla, CA, USA) and were cloned using a Qiagen PCR cloning kit (Qiagen, Tokyo, Japan). Then the cDNA of ALX and 15-LOX were sequenced using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems).

Cryosections of synovium were mounted on silane-coated glass slides and fixed with 4 % (w/v) paraformaldehyde. DIG-labeled antisense riboprobes for human ALX and 15-LOX were prepared by *in vitro* transcription of the pDrive Cloning Vector (Qiagen), which contained human ALX and 15-LOX cDNA[26], and a sense riboprobe prepared in the same way. The synovial sections were treated with 10 µg/ml proteinase K and hybridized with the labeled riboprobes in hybridization solution (Novagen, Madison, WI, USA) for 18 h at 50°C in moistened plastic boxes. After hybridization, the sections were treated with 20 µg/ml RNase A. After extensive washing, the binding of each probe was visualized with an alkaline-phosphate conjugated anti-DIG antibody in 5-bromo-4-chloro-3 indolyl-phosphate and 4-nitroblue tetrazolium chloride solution (Roche Diagnostics GmbH). Then the slides were counterstained with hematoxylin before examination.

Immunohistochemistry for 5-LOX and surface markers. Immunoperoxidase staining was done using a Vectorstain kit according to the manufacturer's protocol[27]. Sections prepared from frozen samples were incubated in methanol containing 3% (v/v) H_2O_2 for 20 min to enhance endogenous peroxidase activity. Then the sections were preincubated in 0.3% (v/v) bovine serum albumin (Sigma-Aldrich Japan K.K., Tokyo, Japan) in phosphate-buffered saline (PBS) for 1 h, followed by incubation with diluted goat serum for 20 min. Subsequently, incubation was done in a humidified chamber for 1 h with an anti-5-LOX antibody (1:100; Research Diagnostics Inc.), anti-CD68 antibody, anti-CD3 antibody, anti-CD4 antibody, anti-CD8 antibody, anti-CD68 antibody, and anti-vWF antibody, or purified normal mouse IgG. After further washing with PBS, sections were incubated with biotinylated goat anti-mouse IgG (Dako) for 30 min, and washed again in PBS. Color was developed by treatment with DBA and the sections were counterstained with hematoxylin.

Statistical analysis. Results are expressed as the mean ± standard deviation. Mean values were compared by the Mann-Whitney test and $p < 0.05$ was considered to indicate a significant difference.

RESULTS

LXA_4 in synovial fluid of patients with RA and OA. The LXA_4 level in synovial fluid from affected joints of patients with RA or OA was measured by ELISA, and an increase of LXA_4 was detected in RA synovial fluid (Figure 1A). The mean concentration of LXA_4 in 30 RA synovial fluid samples was 10.34 ± 14.12 ng/ml, which was significantly greater than in the OA samples (0.66 ± 0.77 ng/ml; $p = 0.0023$). 15-epi-LXA_4 also showed a significantly higher concentration in RA synovial fluids (4.366 ± 4.376 ng/ml) than in OA synovial fluid (0.8553 ± 1.692 ng/ml; $p < 0.0001$; Figure 1B). No significant correlation was found between LXA_4 or 15-epi-LXA_4 levels and patients' clinical features such as sex, age, serum C-reactive protein level, and medications except for prednisolone. As for prednisolone, most patients with RA took it and no patient with OA did. Only 4 patients with RA took aspirin and their 15-epi-LXA_4 levels in synovial fluids were not higher than those without aspirin (3.813 ± 6.192 with aspirin vs 4.451 ± 4.190 without aspirin). In patients with OA, a tendency of high LXA_4 and 15-epi-LXA_4 levels in synovial fluids was detected in patients treated without nonsteroidal antiinflammatory drugs (NSAID; containing aspirin) than those treated with NSAID (for LXA_4, 0.2971 ± 0.1687 with NSAID vs 0.9750 ± 0.9597 without NSAID; for 15-epi-LXA_4, 0.2871 ± 0.2310 with NSAID vs 1.353 ± 2.253 without NSAID), but this difference did not reach statistical significance (p = 0.0820 and 0.2239, respectively). In patients with RA or OA, logarithmic concentrations of LXA_4 were significantly correlated with those of LTB_4 (Figure 1C; Pearson $r = 0.8464$, $p < 0.0001$) or PGE_2 (Figure 1D; Pearson $r = 0.7210$, $p < 0.0001$). Logarithmic concentrations of PGE_2 and LTB_4 were also significantly correlated (Figure 1E; Pearson $r = 0.7931$, $p < 0.0001$). Moreover, significant correlation between logarithmic concentrations of LXA_4 and 15-epi-LXA_4 was detected (Figure 1F; Pearson $r = 0.8119$, $p < 0.0001$).

ALX mRNA expression in synovial tissues of patients with RA or OA. We examined the expression of ALX mRNA in synovial tissues from 20 patients with RA and 10 with OA by RT-PCR and real-time quantitative PCR. Figure 2A shows that ALX mRNA signals were more strongly expressed in the synovial tissue of patients with RA compared to those with OA.

Real-time quantitative PCR revealed 10-fold higher expression of ALX mRNA in RA synovium (9.7 ± 14.48) than OA synovium (1.00 ± 1.01; $p = 0.0165$; Figure 2B).

To determine the distribution of ALX mRNA in the synovial tissues of patients with RA and OA, we performed *in situ* hybridization using DIG-labeled riboprobes. Macrophages were identified by positive staining with anti-CD68 antibody, while fibroblast-like cells were spindle-shaped cells that showed negative staining with anti-CD68 antibody and anti-CD3 antibody. Endothelial cells were identified using anti-vWF antibody. Strong signals for ALX mRNA were seen in macrophages and in a few fibroblast-like cells of the lining layer in patients with RA (Figure 3A, 3B), whereas these cells had faint signals in patients with OA (Figure 3C, 3D).

ALX mRNA expression by synovial fluid cells of patients with RA. Cells in the synovial fluid of 10 patients with RA were used to analyze ALX mRNA expression, revealing 84% ± 5% neutrophils and 16% ± 5% mononuclear cells. ALX mRNA was weakly expressed in cells from the RA synovial fluid by RT-PCR (Figure 2C).

5-LOX and 15-LOX mRNA expression in the synovial tissues of patients with RA or OA. 5-LOX and 15-LOX are the synthetases for LXA_4, and expression of their mRNA in the syn-

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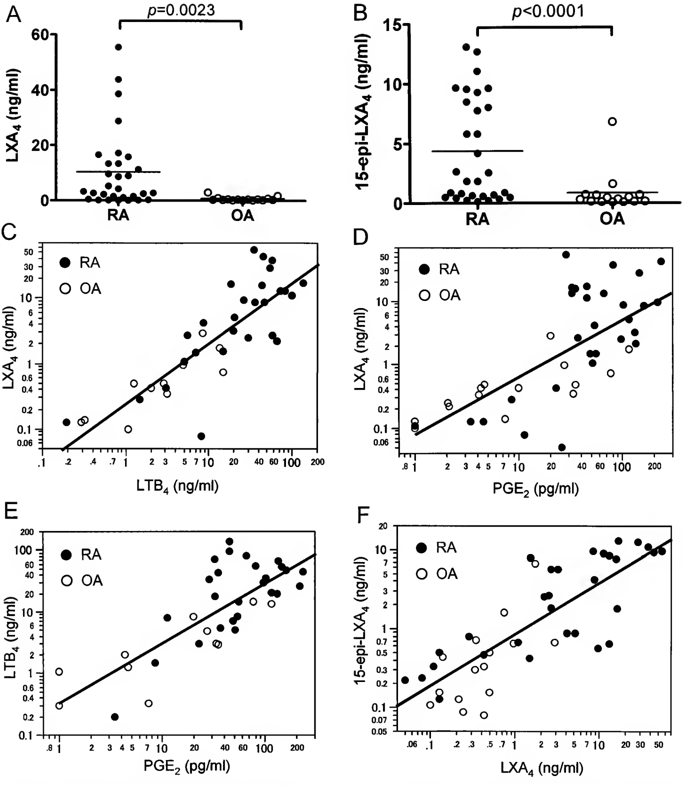

Figure 1. A. Concentration of LXA_4 in synovial fluid of patients with RA or OA. LXA_4 level was significantly higher in RA synovial fluid (10.34 ± 14.12 ng/ml) than OA synovial fluid (0.66 ± 0.77 ng/ml; $p = 0.0023$). B. Concentration of 15-epi-LXA_4 in RA and OA synovial fluids. 15-epi-LXA_4 level was also significantly higher in RA synovial fluid (2.60 ± 0.91 ng/ml) than in OA synovial fluid (0.94 ± 0.92 ng/ml; $p < 0.0001$). Results represent mean ± SD (n = 30 RA, n = 15 OA). C. Double logarithmic plot shows a significant positive correlation between concentrations of LXA_4 and LTB_4 in RA and OA synovial fluids ($r = 0.8464$, $p < 0.0001$). D. Logarithmic concentrations of PGE_2 also correlated with those of LXA_4 ($r = 0.7210$, $p < 0.0001$). E. Logarithmic concentrations of PGE_2 correlated with those of LTB_4 ($r = 0.7931$, $p < 0.0001$). F. Logarithmic concentrations of LXA_4 correlated with those of 15-epi-LXA_4 ($r = 0.8119$, $p < 0.0001$).

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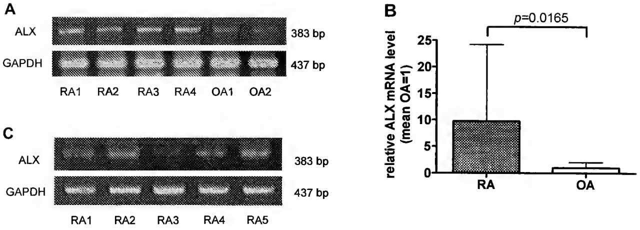

*Figure 2.*A. ALX mRNA expression in synovial tissues from 4 patients with RA and 2 with OA. ALX mRNA was detected by RT-PCR in all synovial samples, but its expression was relatively weak in OA tissues. B. Comparison of the expression of ALX mRNA between synovial tissues from patients with RA and OA. ALX mRNA expression was significantly stronger in RA synovium (9.74 ± 14.48) than in OA synovium (1.00 ± 1.01, $p < 0.0165$). Results represent the mean ± standard deviation (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1. C. ALX mRNA expression in synovial fluid cells from 5 patients with RA. ALX mRNA expression in these cells was weak.

ovial tissues of patients with RA or OA was detected by RT-PCR. Figure 4A shows consistent strong expression of 5-LOX mRNA in RA and OA synovium. Compared with 5-LOX mRNA expression, 15-LOX mRNA expression was weaker in both RA and OA synovium. When 15-LOX mRNA expression in RA and OA synovium was compared by real-time quantitative PCR, its expression was stronger in the synovial tissues of patients with RA (3.01 ± 6.49) compared with those from patients with OA (1.00 ± 1.14), but this difference did not reach statistical significance ($p = 0.1659$; Figure 6B).

In situ hybridization of 15-LOX and immunohistochemical analysis of 5-LOX in synovial tissues of patients with RA. Localization of 5-LOX and 15-LOX in the synovial tissues of patients with RA was analyzed by *in situ* hybridization or immunohistochemistry, respectively. Intense signals for 15-LOX mRNA were detected in macrophages infiltrating RA synovium (Figure 5A, 5B). Immunostaining revealed 5-LOX expression in the synovial lining cells in samples from patients with RA (Figure 5C), as we reported[10].

Quantitative analysis of IL-4 and IL-13 mRNA in synovial tissues of patients with RA and OA. 15-LOX is known to be induced by IL-4[13] and IL-13[14] in human monocytes. When the levels of IL-4 and IL-13 mRNA in synovial tissues from patients with RA and OA were assessed by real-time quantitative PCR, the tissues obtained from patients with RA showed significantly (4-fold) higher expression of IL-4 mRNA (4.16 ± 3.85) than those from patients with OA (1.00 ± 1.10; $p = 0.0060$; Figure 6A). Analysis of IL-13 mRNA also revealed higher expression in the synovial tissues of patients with RA (5.71 ± 7.88) compared with those from patients with OA (1.00 ± 0.69), but this difference was not significant ($p = 0.1659$; Figure 6B).

DISCUSSION

In our study, we detected an increase of LXA_4 and its analog (15-epi-LXA_4) in the synovial fluid as well as increased expression of the LXA_4 receptor (ALX) in synovial tissues of patients with RA compared with those from patients with OA. LXA_4 is an antiinflammatory mediator, and logarithmic concentration of LXA_4 in synovial fluid was positively correlated with that of LTB_4 and PGE_2, which were proinflammatory mediators. Sodin-Semrl, *et al*[28] reported that functioning LXA_4 receptors were expressed by cultured synovial fibroblasts, and suppressed IL-1ß-induced synovial cell activation. In activated synovial fibroblasts, LXA_4 inhibits the synthesis of inflammatory cytokines and matrix metalloproteinases, and also stimulates tissue inhibitor of metalloproteinase-1 production *in vitro*[28]. These findings suggest that LXA_4 is involved in a negative feedback loop that opposes inflammatory cytokine-induced activation of synovial fibroblasts, although other ligands of ALX, for example annexin 1 (also called lipocortin 1) or serum amyloid A, can bind ALX and abrogate inflammation[29-31].

In vivo studies have recently demonstrated that LXA_4 significantly decreases inflammatory infiltrates and edema and has a more potent effect than equimolar concentrations of corticosteroids in mouse and guinea pig models of cutaneous inflammation[32]. Similarly, LXA_4 possesses antiinflammatory effects that may be involved in regulating pathophysiological processes related to the development of inflammatory arthritis such as RA. LXA_4 has a regulatory role in the cytokine network as demonstrated by suppression of tumor necrosis factor-α (TNF-α)-stimulated release of IL-1ß and macrophage inflammatory peptide-2, as well as superoxide production[33]. Numerous types of cells, including neutrophils, monocytes, endothelial cells, and fibroblasts, express high affinity G-protein-coupled receptors (GPCR) for LXA_4 (ALX). Recent

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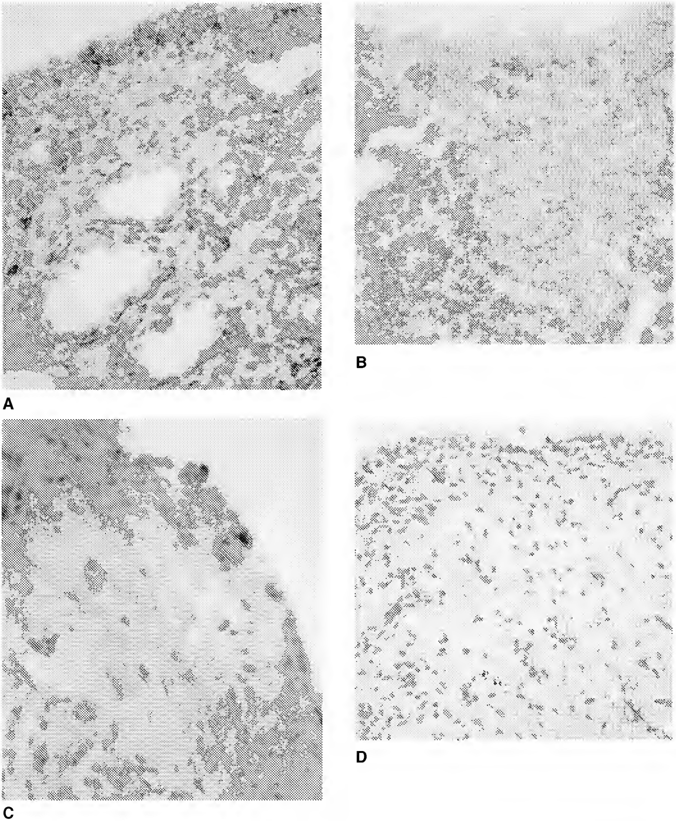

Figure 3. In situ hybridization of ALX mRNA in synovial tissues from patients with RA or OA. *A.* Expression of ALX mRNA was seen in macrophages and fibroblast-like cells from the lining layer of RA synovium. *B.* Negative control: ALX sense probe staining of RA synovium. *C.* Macrophages in OA synovium are weakly positive for ALX mRNA. *D.* Negative control: ALX sense probe staining of OA synovium. *A-D:* Original magnification ×200.

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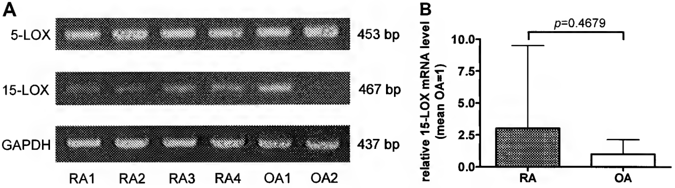

*Figure 4.*A. 5-LOX and 15-LOX mRNA expression in synovial tissues from 4 patients with RA and 2 with OA. 5-LOX and 15-LOX mRNA were detected by RT-PCR in all the samples, but 15-LOX expression was relatively weak compared with that of 5-LOX. B. Comparison of the expression of 15-LOX mRNA in synovial tissues from patients with RA and OA. ALX mRNA expression was stronger in RA syovium (3.01 ± 6.49) than in OA synovium (1.00 ± 1.14, p = 0.4679). Results represent the mean ± SD (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1.

nomenclatures have clarified human ALX, which was at first recognized as formyl peptide receptor-like 1 (FPRL1)[34,35]. FPRL1 is known to possess high DNA sequence homology (~70%) to human formyl peptide receptor (FPR), which is a low affinity receptor for LXA_4. FPRL2 is also one of the FPR family receptors, but LXA_4 has not been confirmed as a ligand for FPRL2. Although our primer sets for ALX cannot distinguish ALX from FPR or FPRL2, ALX is the only high affinity receptor for LXA_4. ALX transgenic mice showed diminished activation of the proinflammatory transcription factor nuclear factor-kappa B (NF-κB) in the local inflammatory response[36]. Our data suggest that high levels of proinflammatory lipid mediators (PGE_2 and LTB_4) induce production of LXA_4 and 15-epi-LXA_4 for antiinflammation (Figure 1C, 1D, 1E, 1F)[4].

Biosynthesis of leukotrienes is initiated by insertion of molecular oxygen at the carbon-5 position of arachidonic acid. Insertion of molecular oxygen at the carbon-5 and carbon-15 positions by 5-LOX and 15-LOX, respectively, results in the formation of lipoxins. This can occur through oxygenation of leukocyte-derived LTA_4 by platelet lipoxygenase through cell–cell interactions[37]. Several reports have indicated that lipoxygenase can be found in synovial tissues from patients with RA.

Bonnet, *et al* described the expression of 5-LOX and 5-lipoxygenase-activating protein (FLAP) mRNA in cultured human synovial cells[9] and our group has reported the presence of 5-LOX in the synovial lining layer[10]. In our study we demonstrated more highly expressed 15-LOX mRNA in synovial tissues from patients with RA than in those from patients with OA. The route of lipoxin formation depends on the cells and enzymes present and can be modulated by cytokines. IL-4[13] and IL-13[14], which are thought to be negative regulators of the inflammatory response, increase 15-LOX expression and activity as does PGE_2[4], thereby enhancing LXA_4 formation. The generation of lipoxins by both proinflammatory and antiinflammatory mediators may lead to negative feedback inhibition of the inflammatory response and thus protect the host from potentially deleterious neutrophil-induced responses. 15-LOX was more strongly expressed in the synovial lining of patients with RA than that of patients with OA (Figure 4B). In patients with RA, IL-13 mRNA was also detectable in synovial fluid mononuclear cells and synovial tissues, and the IL-13 level in synovial fluids was significantly higher than that reported for IL-4[38]. 5-LOX was constitutively expressed in both RA and OA synovial tissues[10]. These data suggest that 15-LOX induced by IL-13 may be one of the regulators of the production of LXA_4 in inflamed joints. A wide distribution of LXA_4 levels in synovial fluids might depend on that of 15-LOX. Moreover, overexpression of leukotriene B_4 receptor 2 (BLT2) in synovial leukocytes of patients with RA[10] could contribute to the upregulation of IL-13[39].

One of the pathways identified for LXA_4 biosynthesis involves platelet–neutrophil interactions[37]. This pathway has been highlighted as a route of LXA_4 formation within the vasculature that involves 5-LOX in leukocytes and 12-LOX in platelets. There are numerous platelets in synovial joints, so platelets in synovilal fluid might also be involved in LXA_4 formation.

In synovial fluids of patients with RA, 15-epi-LXA_4 was detected at about one-quarter the level of LXA_4. The concentration of 15-epi-LXA_4 in synovial fluids was around 10 times that in plasma (0.25 ± 0.63 ng/ml) in a report by Chiang, *et al*[40]. Acetylation of cyclooxygenase-2 by aspirin could lead to transcellular biosynthesis of epi-lipoxin, the so-called aspirin-triggered lipoxins. Recently, in a randomized, placebo-controlled study of 8 weeks, a clinically relevant dose of aspirin was found to increase antiinflammatory 15-epi-LXA_4. Plasma 15-epi-LXA_4 levels at 8 weeks were significantly greater than those before aspirin treatment[40]. In our study we were not able to detect the relation between aspirin taking and concentrations of 15-epi-LXA_4 in synovial fluids of patients with RA and OA. On the contrary, LXA_4 and 15-epi-LXA_4 levels were higher in OA patients without NSAID compared with those

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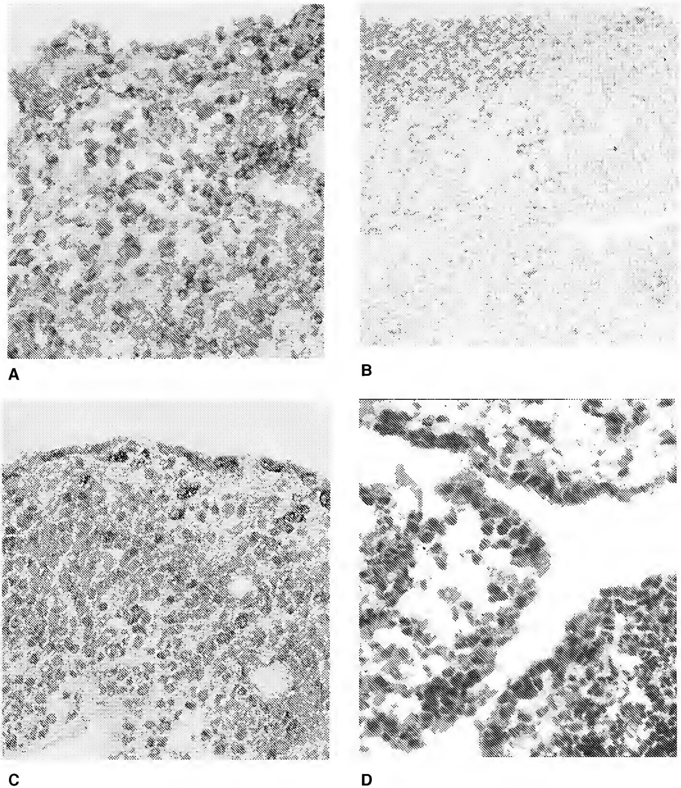

Figure 5. In situ hybridization of 15-LOX mRNA and immunohistochemical analysis of 5-LOX in synovial tissues from patients with RA. A. Expression of 15-LOX mRNA is seen in macrophages from the lining layer (original magnification ×200). B. Negative control: 15-LOX sense probe staining (original magnification ×100). C. Synovial lining cells stained for 5-LOX (original magnification ×200). D. Distribution of CD68-positive cells. Macrophages show staining by the anti-CD68 antibody (original magnification ×200).

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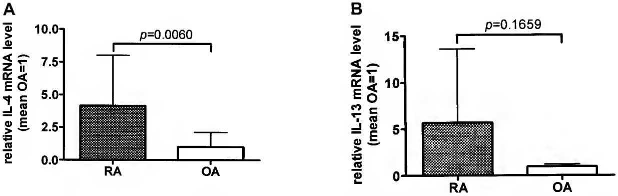

Figure 6. IL-4 and IL-13 mRNA expression in synovial tissues from patients with RA and OA. A. IL-4 mRNA expression was significantly stronger in RA syovium (4.16 ± 3.85) than in OA synovium (1.00 ± 1.10, p = 0.0060). B. IL-13 mRNA expression was also stronger in RA syovium (5.71 ± 7.88) than in OA synovium (1.00 ± 0.69, p = 0.1659). Results represent the mean ± SD (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1.

treated with NSAID, although 15-epi-LXA_4 levels in synovial fluid can differ from those in plasma. On the other hand HMG-CoA reductase inhibitor drugs, the so-called statins, were recently thought to generate 15-epi-LXA_4[41], only one patient took statin in this study. Regulatory systems of lipoxins, especially 15-epi-LXA_4, remain to be elucidated.

Biosynthesis of lipid mediators has been observed to show a biphasic pattern during the inflammatory response. *In vivo* analysis of eicosanoid formation in a murine dorsal air-pouch model of inflammation has revealed a distinct time-dependent profile[42]. TNF–induced neutrophil accumulation within the dorsal pouch coincides with an increase of intradorsal PGE_2 levels. A persistent and marked increase of LXA_4 levels occurred, along with a reduction of neutrophil infiltration and PGE_2 production. Our synovial fluid analysis indicated that a patient with high levels of PGE_2 and LTB_4 had high levels of LXA_4 and 15-epi-LXA_4. Lee, *et al* reported that LXA_4 was detected in bronchoalveolar lavage fluid samples from 9 of 12 patients with lung disease and not detected in healthy control samples[43]. Although another report demonstrated that bronchoalveolar lavage fluids in patients with scleroderma lung disease had low levels of LXA_4 and high levels of PGE_2 because of impaired stimulatory effect of PGE_2 on 15-LOX[44], our results support that LXA_4 is basically produced responding to PGE_2 in order to degrade the inflammation. It seems possible that PGE_2 induces a switch in lipid mediator synthesis from predominance of 5-LOX-yielding LTB_4 to predominance of 15-LOX-generating LXA_4, a response that would be paralleled by a reduction of neutrophil infiltration[4]. This leads to the proposal that biosynthesis of inflammatory lipid mediators is biphasic, with a role for eicosanoids in the initiation, progression, and termination of the inflammatory response. The initial phase is coupled to leukotriene biosynthesis, while subsequent prostaglandin formation is coupled to induction of lipoxygenase activity and lipoxin biosynthesis, promoting the resolution of inflammation.

Our study demonstrated that LXA_4 was synthesized in the synovial tissues of patients with RA via a transcellular pathway, which might be regulated by IL-13-induced 15-LOX. LXA_4 and its analog can act as an antiinflammatory negative feedback system for proinflammatory mediators such as LTB_4 and PGE_2 that are involved in the pathogenesis of RA. Activation of lipoxin synthesis, for example by augmentation of 15-LOX, could be a potential new therapeutic approach for RA.

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